Effects of common mutations in the SARS-CoV-2 Spike RBD domain and its ligand the human ACE2 receptor on binding affinity and kinetics

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Abstract

The interaction between the SARS-CoV-2 virus Spike protein's receptor binding domain (RBD) and the ACE2 cell surface protein is required for viral infection of cells. Mutations in the RBD domain are present in SARS-CoV-2 variants of concern that have emerged independently worldwide. For example, the more transmissible B.1.1.7 lineage has a mutation (N501Y) in its Spike RBD domain that enhances binding to ACE2. There are also ACE2 alleles in humans with mutations in the RBD binding site. Here we perform a detailed affinity and kinetics analysis of the effect of five common RBD mutations (K417N, K417T, N501Y, E484K and S477N) and two common ACE2 mutations (S19P and K26R) on the RBD/ACE2 interaction. We analysed the effects of individual RBD mutations, and combinations found in new SARS-CoV-2 variants first identified in the UK (B.1.1.7), South Africa (B.1.351) and Brazil (P1). Most of these mutations increased the affinity of the RBD/ACE2 interaction. The exceptions were mutations K417N/T, which decreased the affinity. Taken together with other studies, our results suggest that the N501Y and S477N mutations primarily enhance transmission, the K417N/T mutations facilitate immune escape, and the E484K mutation facilitates both transmission and immune escape.

Introduction

Since its identification in 2019, the second coronavirus able to induce a severe acute respiratory syndrome in humans, SARS-CoV-2, has resulted in the most severe global pandemic in 100 years. To date more than 135 million people have been infected, resulting in the deaths from the resulting disease, COVID-19, of more than 3 million people ("WHO Coronavirus (COVID-19) Dashboard," 2021), and measures introduced to control spread have had harmful social and economic impacts. Fortunately, effective vaccines have been developed, and a global vaccination programme is underway (Mahase, 2021). New SARS-CoV-2 variants of concern are emerging that are making containment of the pandemic more difficult, by increasing transmissivity of the virus (Davies and Edmunds, 2021; Korber et al., 2020; Volz et al., 2021a, 2021b; Washington et al., 2021) and/or its resistance to protective immunity induced by previous infection or vaccines (Darby and Hiscox, 2021; Dejnirattisai et al., 2021; Garcia-Beltran et al., 2021; Madhi et al., 2021a, 2021b; Mahase, 2021).(Volz et al., 2021a, 2021b)

The SARS-CoV-2 virus enters cells following an interaction between the Spike (S) protein on its surface with angiotensin-converting enzyme 2 (ACE2) on cell surfaces (V'kovski et al., 2021). The receptor binding domain (RBD) of the Spike protein binds the membrane-distal portion of the ACE2 protein. The S protein forms a homotrimer, which is cleaved shortly after synthesis into two fragments that remain associated non-covalently: S1, which contains the RBD, and S2, which mediates membrane fusion following the binding of Spike to ACE2 (V'kovski et al., 2021). During the pandemic mutations have appeared in the Spike protein that apparently increase transmissivity (Davies and Edmunds, 2021; Korber et al., 2020; Volz et al., 2021a, 2021b; Washington et al., 2021). One that emerged early in Europe, D614G, and quickly became dominant globally (Korber et al., 2020), increases the density of intact Spike trimer on the virus surface by preventing premature dissociation of S1 from S2 following cleavage (Zhang et al., 2021, 2020). A later mutant, N501Y, which has appeared in multiple lineages, lies within the RBD domain, and increases its affinity for ACE2 (Starr et al., 2020; Supasa et al., 2021). These findings suggest that mutations that directly or indirectly enhance Spike binding to ACE2 will increase transmissivity.

Prior infection by SARS-CoV-2 and current vaccines induce antibody responses to the Spike protein, and most neutralizing antibodies appear to bind to the Spike RBD domain (Garcia-

50 Beltran et al., 2021; Greaney et al., 2021a; Rogers et al., 2020). Some variants of concern
51 have mutations in their RBD domain that confer resistance to neutralizing antibodies (Darby
52 and Hiscox, 2021; Dejnirattisai et al., 2021; Garcia-Beltran et al., 2021; Madhi et al., 2021a,
53 2021b; Mahase, 2021). What is less clear is the precise effect of these mutations on the
54 affinity and kinetics of the binding of RBD to ACE2. Previous studies of the interaction
55 between the Spike RBD and ACE2 have produced a wide range of affinity and kinetic
56 estimates under conditions (e.g. temperature) that are not always well defined (Lei et al.,
57 2020; Shang et al., 2020; Supasa et al., 2021; Wrapp et al., 2020; Zhang et al., 2021, 2020).
58 Precise information is needed to assess the extent to which RBD mutations have been
59 selected because they enhance ACE2 binding or facilitate immune evasion.

60 In this study we undertook a detailed affinity and kinetic analysis of the interaction between
61 Spike RBD and ACE2 at physiological temperatures, taking care to avoid common pitfalls.
62 We used this optimized approach to analyse the effect of important common mutations
63 identified in variants of RBD and ACE2. Both mutations of ACE2 (S19P, K26R) and most of
64 the mutations of RBD (N501Y, E484K, and S477N) enhanced the interaction, with some RBD
65 mutations (N501Y) increasing the affinity by ~10 fold. Increased binding was the result of
66 decreases in dissociation rate constants (N501Y, S477N) and/or increases in association rate
67 constants (N501Y, E484K). Although the K417N/T mutations found in the South African
68 (B.1.351) and Brazilian (P.1) variants both decreased the affinity, the affinity-enhancing
69 N501Y and E484K mutations that are also present in both variants confer a net ~4 fold
70 increase in the affinity of their RBD domains for ACE2.

71 Results

72 Selection of variants

73 The focus of this study was to analyse common and therefore important variants of RBD and
74 ACE2. Henceforth we will refer to the common ACE2 allele and RBD of the original SARS-
75 CoV-2 strain sequenced in Wuhan as wild-type (WT). We chose mutations of RBD within the
76 ACE2 binding site that have appeared independently in multiple SARS-CoV-2 lineages/clades
77 (Fig. 1 and Fig. S1) (Hodcroft, 2021; Rambaut et al., 2020), suggesting that they confer a
78 selective advantage, rather than emerged by chance, such as through a founder effect. The
79 N501Y mutation has appeared in the B.1.1.7 (20I/501Y.V1), B.1.351 (20H/501Y.V2), and P.1

lineages (20J/501Y.V3) first identified in the UK, South Africa and Brazil, respectively. The E484K mutation is present in the B.1.351 and P.1 lineages, and has appeared independently in many other lineages including P.2 (20B/S.484K), B.1.1.318, B.1.525 (20A/S:4.4K), and B.1.526 (20C/S.484K). E484K has also appeared in VOC-202102-02, a subset of the B.1.1.7 lineage identified in the UK("SARS-CoV-2 Variants of concern and variants under investigation - GOV.UK," 2021) . The S477N mutation became dominant for periods in Australia (clade 20F) and parts of Europe (20A.EU2), and then appeared in New York in lineage B.1.526 (H. Zhou et al., 2021). Mutations of K417 have appeared independently in the South African B.1.351 and Brazilian P.1 lineages. Interestingly, N501Y, E484K and S477N were the main mutations that appeared following random RBD mutagenesis and in vitro selection of mutants with enhanced ACE2 binding (Zahradník et al., 2021).

We selected for analysis the two most common mutations of ACE2 within the RBD binding site, K26R and S19P (Fig. 1C). They are present in 0.4% and 0.03%, respectively, of all samples in the gnomAD database (Karczewski et al., 2020), while other ACE2 mutations in the RBD binding site are much less frequent (<0.004%). K26R is observed in all the major gnomAD populations but is most common in Ashkenazi Jews (1%), and (non-Finnish) north-western Europeans (0.6%). It is less common in Africans/African-Americans and South Asians (0.1%) and rare in Finnish (0.05%) and East-Asian (0.001%) populations. The S19P mutant is almost exclusively found in Africans/African-Americans (0.3 %).

Measurement of affinity and kinetics

To measure the effects of these mutations on the affinity and kinetics of the RBD/ACE2 interaction we used surface plasmon resonance, which allows very accurate measurements, provided that common pitfalls are avoided, particularly protein aggregation, mass-transport limitations and rebinding (Merwe and Barclay, 1996; Myszka, 1997). Monomeric, soluble forms of the ectodomain of the ACE2 and the Spike RBD-domain were expressed in human cells, to retain native glycosylation, and purified (Fig. S2). ACE2 was captured onto the sensor surface via a carboxy-terminal biotin and RBD injected over the ACE2 at different concentrations (Fig. 2A). Excellent fits of 1:1 Langmuir binding model to the data yielded an association rate constant (k_{on}) of 0.9 ± 0.05 $\mu M^{-1}.s^{-1}$ and a dissociation rate constant (k_{off}) of 0.067 ± 0.0011 s^{-1} (mean ± SD, n=6, Table 1). These rate constants are 3 to 25 fold faster than previously reported for the same interaction (Lei et al., 2020; Shang et al., 2020;

111 Supasa et al., 2021; Wrapp et al., 2020; Zhang et al., 2021). However, previous experiments
112 were conducted at unphysiologically low temperatures (i.e. below 37° C) and under
113 conditions in which mass-transport limitations and rebinding are highly likely (see
114 Discussion). These factors, and the presence of protein aggregates, would all lower the
115 measured rate constants. In contrast, our measurements were conducted at 37° C and
116 under conditions in which mass-transfer limitation and rebinding were excluded. The latter
117 is demonstrated by the fact that measured k_{on} and k_{off} rates were clearly maximal at the low
118 level of ACE2 immobilization (~50 RU) used in our experiments (Fig. 2B and C). The excellent
119 fit of the 1:1 binding model to our data excludes an effect of protein aggregates, which yield
120 complex kinetics. The calculated dissociation constant (K_D) was 74 ± 4 nM (mean ± SD, n=6,
121 Table 1). We also measured K_D by equilibrium binding (Fig. 2D), which avoids any artefacts
122 induced by mass transfer limitations and rebinding. This K_D determined by equilibrium
123 binding was very similar to the value calculated from kinetic data [63 ± 7.7 nM (mean ± SD,
124 n = 24, Table 1], and did not vary with immobilization level (Fig. 2E), further validating our
125 kinetic measurements. These affinity values are within the wide range reported in previous
126 studies, which varied from K_D 11 to 133 nM (Lei et al., 2020; Shang et al., 2020; Supasa et al.,
127 2021; Wrapp et al., 2020; Zhang et al., 2021).

128 **The effect of RBD mutations**

129 We next evaluated the effect of RBD mutations on the affinity and kinetics of binding to
130 ACE2 (Figure 3 and Table 1). Example sensorgrams are shown of mutations that increased
131 (N501Y, Fig. 3A) or decreased (K417N, Fig. 3B) the binding affinity, while the key results
132 from all mutants are summarized in Figure 3C. The single mutations S477N, E484K and
133 N501Y all enhanced binding. The N501Y mutation had the biggest effect, increasing the
134 affinity ~10 fold to K_D ~7 nM, by increasing the k_{on} ~1.8 fold and decreasing the k_{off} by ~ 7
135 fold. The S477N and E484K mutations increased the affinity more modestly (~ 1.5 fold), by
136 decreasing the k_{off} (S477N) or increasing the k_{on} (E484K). The K417T and K417N mutations
137 decreased the affinity ~2 and ~4 fold, respectively, mainly by decreasing the k_{on} but also by
138 increasing the k_{off}. Affinity-altering mutations in binding sites mainly affect the k_{off} (Agius et
139 al., 2013), and have more modest effects on the k_{on}. Changes in electrostatic interactions
140 can dramatically affect the k_{on} (Schreiber and Fersht, 1996), and are a plausible explanation
141 for the effects of the mutations K417T, K417N and E484K on k_{on}. K417 forms a salt bridge

142 with D30 on ACE2 (Lan et al., 2020) while E484 is ~9 Å from E75 on ACE2 (Lan et al., 2020).
143 Thus the mutations K417N/T and E484K would decrease and increase, respectively, long-
144 range electrostatic forces that may accelerate association (Schreiber and Fersht, 1996).

145 We also examined the effect on ACE2 binding of the combinations of RBD mutations,
146 including combinations present in VOC-202102-02, a subset of the B.1.1.7 lineage (N501Y)
147 with the E484K mutation ("SARS-CoV-2 Variants of concern and variants under investigation
148 - GOV.UK," 2021), and the B.1.351 and P.1 variants (Fig. 3C, Table 1). In the case of VOC-
149 202102-02, the addition of the E484K mutation to N501Y further increased the affinity, to
150 ~15 fold higher than WT RBD (K_D ~5 nM), by further increasing the k_{on}. Because the higher
151 k_{on} could result in mass transfer limiting binding, we confirmed that the kinetic
152 measurement for this variant was not substantially affected by varying levels of
153 immobilization (Fig. S4). The affinity of the B.1.351 (K417N/ E484K/N501Y) and P.1
154 (K417T/E484K/N501Y) RBD variants for ACE2 increased by 3.7 and 5.3 fold, respectively,
155 relative to wild type RBD by increasing the k_{on} and decreasing the k_{off} rate constants.

156 We next examined whether the effects of the mutations were additive, as is typically the
157 case for multiple mutations at protein/protein interfaces (Wells, 1990). To do this we
158 converted the changes in K_D to changes in binding energy ($\Delta\Delta G$, Table 2) and examined
159 whether the $\Delta\Delta G$ measured for RBD variants with multiple mutations was equal to the sum
160 of the $\Delta\Delta G$ values measured for the individual RBD mutants. This was indeed the case (Fig.
161 3D), indicating that the effects on each mutation are independent. This is consistent with
162 them being spaced well apart within the interface (Fig. 1C), and also validates the accuracy
163 of the affinity measurements.

164 **The effects of ACE2 mutations**

165 We next examined the effects of mutations of ACE2 (S19P and K26R) on binding to both wild
166 type and common variants of RBD (Fig. 4 and Table 1). Both S19P and K26R increased the
167 affinity of WT RBD binding by ~3.7 and ~2.4 fold (Fig. 4A). These increases in affinity were
168 the result of increases in the k_{on} and decreases in the k_{off}.

169 Finally, we looked for interactions between RBD and ACE2 mutations by measuring the
170 effects of the ACE2 mutations on binding to all mutant forms of RBD (Table 1). After
171 converting changes in K_D to $\Delta\Delta G$ (Table 2) we examined whether $\Delta\Delta G$ measured for a given

172 ACE2 variant/RBD variant interaction was equal to the sum of the ΔΔG measured for ACE2
173 variant/RBD WT and ACE WT/RBD variant interactions. This is depicted as the difference
174 between the measured and predicted ΔΔG for interactions between ACE2 and RBD variants
175 (ΔΔΔG in Figs. 4B and C). In most cases ΔΔΔG values were close to zero, indicating that the
176 effects of these mutations largely independent. The one notable exception was the ACE2
177 S19P and RBD S477N variants, where the measured value was significantly lower than the
178 predicted value (Fig. 4B), indicating that these mutations were not independent. This is
179 consistent with the fact that the ACE2 residue S19 is adjacent to RBD residue S477 in the
180 contact interface (Fig. 1C).

181 **Discussion**

182 While our finding that the SARS-CoV-2 RBD binds ACE2 with an affinity of K_D 74 nM at 37°C
183 is consistent with previous studies (K_D 11 to 133 nM) (Lei et al., 2020; Shang et al., 2020;
184 Supasa et al., 2021; Wrapp et al., 2020; Zhang et al., 2021, 2020), the rate constants that we
185 measured (k_{on} 0.9 $\mu M^{-1}.s^{-1}$ and k_{off} 0.067 s^{-1}) were more than 3 fold faster than all previous
186 reports. One likely reason for this is that previous measurements were performed at lower
187 temperatures, which almost always decreases rate constants. While one study stated that
188 binding constants were measured at 25°C (Zhang et al., 2020), most studies did not report
189 the temperature, suggesting that they were performed at room temperature, or the
190 standard instrument temperature (20-25°C). A second likely reason is that previous kinetic
191 studies were performed under conditions in which the rate of diffusion of soluble molecule
192 to the sensor surface limits the association rate and rebinding of dissociated molecules to
193 the surface reduces the measured dissociation rate. These are known pitfalls of both
194 techniques used in these studies, which were surface plasmon resonance (Myszka, 1997)
195 and bilayer interferometry (Abdiche et al., 2008). In the present study we avoided these
196 issues by immobilizing a very low level of ligand on the sensor surface. A third possible
197 reason is that the proteins were aggregated, which can cause problems even when
198 aggregates are a very minor contaminant (van der Merwe and Barclay, 1996). The presence
199 of aggregates results in complex binding kinetics, which can be excluded if the simple 1:1
200 Langmuir binding model fits the kinetic data. While this was demonstrated in the present
201 study, and some previous studies (Shang et al., 2020; Wrapp et al., 2020; Zhang et al., 2021),

202 such fits were not shown in other studies, one of which reported more than 20 fold slower
203 kinetics than reported here (Lei et al., 2020; Supasa et al., 2021).

204 The RBD mutants that we selected for analysis have all emerged independently and become
205 dominant in a region at least once in different lineages, suggesting that they provide a
206 selective advantage. Our finding that the N501Y, E484K, and S477N all increase the binding
207 affinity of RBD for ACE2 raises the question as to whether this contributed to their selection.
208 Several lines of evidence suggest that enhancing the Spike/ACE2 interaction would be
209 advantageous. Firstly, the virus has spread only very recently to humans from another
210 mammalian host, providing insufficient time for optimization of the affinity. Secondly,
211 epidemiological studies have demonstrated enhanced transmissibility of the B.1.1.7 variant,
212 which has the N501Y mutation (Volz et al., 2021b; Washington et al., 2021). Finally, a SARS-
213 CoV-2 variant with the Spike mutation D614G, which increases its activity by stabilizing it
214 following furin cleavage (Zhang et al., 2021, 2020), rapidly became dominant globally after it
215 emerged (Korber et al., 2020; Volz et al., 2021a). Taken together, these findings suggest that
216 WT Spike/ACE2 interaction is limiting for transmission, and that mutations which enhance it,
217 including the N501Y, E484K, and S477N mutations, would provide a selective advantage by
218 increasing transmissibility. This raises two questions. Firstly, will other RBD mutations
219 appear in SARS-CoV-2 which further enhance transmission? This seems likely, given that a
220 large number of RBD mutations have been identified that increase the RBD/ACE2 affinity
221 (Starr et al., 2020; Zahradník et al., 2021). Secondly, will combinations of existing mutations
222 be selected because they further increasing the affinity? While the appearance E484K
223 together with the N501Y in three lineages (B.1.1.7, B.1.351 and P.1) supports this, it is also
224 possible that E484K was selected because it disrupts antibody neutralization, as discussed
225 below.

226 Studies of other enveloped viruses, including SARS-CoV-2, suggest that increases in affinity
227 of viral fusion ligands for their cellular receptors can increase cell infection and disease
228 severity (Hasegawa et al., 2007; Li et al., 2005). One study found that increasing this affinity
229 enabled the virus to infect cells with lower receptor surface density (Hasegawa et al., 2007).
230 It follows that increases in affinity could increase the number of host tissues infected, which
231 could increase the severity of disease (Cao and Li, 2020) and/or increase the viral load in the

232 upper respiratory tract el (Hoffmann et al., 2020; Wölfel et al., 2020), thereby increasing
233 spread.

234

235 Another mechanism by which mutations of RBD could provide a selective advantage is
236 through evasion of immune responses. This is supported by the observation that
237 neutralizing antibodies present in those infected by or vaccinated against SARS-CoV-2
238 primarily target the RBD domain (Garcia-Beltran et al., 2021; Greaney et al., 2021a; Rogers
239 et al., 2020). Furthermore, two variants with RBD mutations that abrogate antibody
240 neutralization, B.1.351 and P1, became dominant in regions with very high levels of prior
241 SARS-CoV-2 infection (Cele et al., 2021; Dejnirattisai et al., 2021; Hoffmann et al., 2021;
242 Sabino et al., 2021; Tegally et al., 2021; D. Zhou et al., 2021). Both of these lineages include
243 the N501Y mutation, but this appears to have fairly modest effect on antibody
244 neutralization (Greaney et al., 2021a, 2021b). In contrast, the E484K mutation, also present
245 in both lineages, potently disrupts antibody neutralization (Greaney et al., 2021a, 2021b).
246 Our finding that the K417N/T mutants present in B.1.351/P.1 lineages decrease the affinity
247 of RBD for ACE2 suggests that they were selected because they facilitate immune escape.
248 Indeed, mutations of K417 can block antibody neutralization, albeit less effectively than
249 E484K (Greaney et al., 2021a, 2021b; Wang et al., 2021). It is notable that these affinity-
250 reducing K417N/T mutants have only emerged together with mutants (N501Y and E484K)
251 that increase the affinity of RBD for ACE2, suggest a cooperative effect between mutations
252 that enhance immune escape and mutations that increase affinity.

253 The effect of the increased affinity for SARS-CoV-2 Spike RBD of the K26R and S19P ACE2
254 mutants are less clear. The evidence summarised above that WT RBD/ACE2 binding is
255 limiting for SARS-CoV-2 transmission, suggest that carriers of these ACE2 variants will be at
256 greater risk of infection and/or severe disease. In contrast to SARS-CoV-2 RBD mutations,
257 the effects of ACE2 variants are primarily relevant to the carriers of these mutations. A
258 preliminary analysis (MacGowan et al., 2021) suggests that the carriers of the K26R ACE
259 allele might be at increased risk of severe disease, but the findings did not reach statistical
260 significance, and further studies are required.

261 The interaction that we identified between the RBD S477N and ACE2 S19P mutants
262 highlights the importance of considering variation in the host population when studying the

263 evolution of viral variants. In this case, the opposite effect of the RBD S477N mutation on its
264 affinity for ACE2 S19P (decreased) compared with ACE2 WT (increased), suggests that this
265 RBD variant may have a selective disadvantage amongst carriers of the ACE2 S19P variant, in
266 contrast to those with WT ACE2, where it is advantageous. However, the low frequency of
267 this variant will make this difficult to detect and suggests that it is unlikely to be important
268 at a population level.

269 It is noteworthy that the two most common ACE2 variants are in positions on ACE2 with no
270 known functional activity. This raises the question as to whether these mutations are a
271 remnant of historic adaption to pathogens that utilised this portion of ACE2. The fact that
272 ACE2 S19P mutation is largely confined to African/African-American populations, suggests
273 that it is more recent than K26R and/or selected by pathogen(s) confined to the African
274 continent.

275 Materials and Methods

276 ACE2 and RBD variant constructs

277 The soluble WT ACE2 construct, which was kindly provided by Ray Owens (Oxford Protein
278 Production Facility-UK), encoded the following protein:

279 STIEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDKWSAFLKEQSTLAQMYPLQ
280 EIQNLTVKLQLQALQQNGSSVLSEDKSKRLNTILNTMSTIYSTGKVCNPDNPQECLLLEPGLNEIMANSLD
281 YNERLWAWESWRSEVGKQLRPLYEEYVVLKNEMARANHYEDYGDYWRGDYEVNGVDGYDYSRGQLI
282 EDVEHTFEEIKPLYEHLHAYVRAKLMNAYPSYISPIGCLPAHLLGDMWGRFWTNLYSLTVPFGQKPNIDV
283 TDAMVDQAWDAQRIFKEAEKFFVSVGLPNMTQGFWENSMLTDPGNVQKAVCHPTAWDLGKGDFRI
284 LMCTKVTMDDFLTAHHEMGHIQYDMAYAAQPFLLRNGANEGFHEAVGEIMSLSAATPKHLKSIGLLSP
285 DFQEDNETEINFLLKQALTIVGTLPFTYMLEKWRWMVFKGEIPKDQWMKKWWEMKREIVGVVEPVP
286 HDETYCDPASLFHVSNDYSFIRYYTRTLYQFQFQEALCQAAKHEGPLHKCDISNSTEAGQKLFNMLRLGK
287 SEPWTLALENVVGAKNMNVRPLLNYFEPLFTWLKDQNKNSFVGWSTDWSPYADLNDIFEAQKIEWHE
288 KHHHHHH

289 The carboxy-terminal end has a biotin acceptor peptide (underlined) followed by an
290 oligohistidine tag.

291 The WT RBD construct, which was kindly provided by Quentin Sattentau (Sir William Dunn
292 School of Pathology), encoded the following protein:

293 RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLND
294 LCFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKS
295 NLKPFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKS
296 TNLVKNKCVNFHHHHHH

297 The carboxy-terminal end has an oligohistidine tag.

298 ACE2 and RBD point mutations were introduced using the Agilent QuikChange II XL Site-
299 Directed Mutagenesis Kit following the manufacturer's instructions. The primers were
300 designed using the Agilent QuikChange primer design web program.

301 **HEK293F cell transfection**

302 Cells were grown in FreeStyle™ 293 Expression Medium (12338018) in a 37 °C incubator
303 with 8% CO_2 on a shaking platform at 130 rpm. Cells were passaged every 2-3 days with the
304 suspension volume always kept below 33.3% of the total flask capacity. The cell density was
305 kept between 0.5 and 2 million per ml. Before transfection cells were counted to check cell
306 viability was above 95% and the density adjusted to 1.0 million per ml. For 100 ml
307 transfection, 100 µl FreeStyle™ MAX Reagent (16447100) was mixed with 2 ml Opti-MEM
308 (51985034) for 5 minutes. During this incubation 100 µg of expression plasmid was mixed
309 with 2 ml Opti-MEM. For in situ biotinylation of ACE2 90 µg of expression plasmid was
310 mixed with 10 µg of expression plasmid encoding the BirA enzyme. The DNA was then
311 mixed with the MAX Reagent and incubated for 25 minutes before being added to the cell
312 culture. For ACE2 in situ biotinylation, biotin was added to the cell culture at a final
313 concentration of 50 µM. The culture was left for 5 days for protein expression to take place.

314 **Protein purification**

315 Cells were harvested by centrifugation and the supernatant collected and filtered through a
316 0.22 µm filter. Imidazole was added to a final concentration of 10 mM and PMSF added to a
317 final concentration of 1 mM. 1 ml of Ni-NTA Agarose (30310) was added per 100 ml of
318 supernatant and the mix was left on a rolling platform at 4 °C overnight. The supernatant
319 mix was poured through a gravity flow column to collect the Ni-NTA Agarose. The Ni-NTA
320 Agarose was washed 3 times with 25 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and
321 20 mM imidazole at pH 8). The protein was eluted from the Ni-NTA Agarose with elution
322 buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole at pH 8). The protein was

323 concentrated, and buffer exchanged into size exclusion buffer (25 mM NaH_2PO_4, 150 mM
324 NaCl at pH 7.5) using a protein concentrator with a 10,000 molecular weight cut-off. The
325 protein was concentrated down to less than 500 µl before loading onto a Superdex 200
326 10/300 GL size exclusion column (Fig. S2). Fractions corresponding to the desired peak were
327 pooled and frozen at -80 °C. Samples from all observed peaks were analysed on an SDS-
328 PAGE gel (Fig. S2).

329 **Surface plasmon resonance (SPR)**

330 RBD binding to ACE2 was analysed on a Biacore T200 instrument (GE Healthcare Life
331 Sciences) at 37°C and a flow rate of 30 µl/min. Running buffer was HBS-EP (BR100669).
332 Streptavidin was coupled to a CM5 sensor chip (29149603) using an amine coupling kit
333 (BR100050) to near saturation, typically 10000-12000 response units (RU). Biotinylated
334 ACE2 WT and variants were injected into the experimental flow cells (FC2–FC4) for different
335 lengths of time to produce desired immobilisation levels (40–800 RU). FC1 was used as a
336 reference and contained streptavidin only. Excess streptavidin was blocked with two 40 s
337 injections of 250 µM biotin (Avidity). Before RBD injections, the chip surface was
338 conditioned with 8 injections of the running buffer. A dilution series of RBD was then
339 injected simultaneously in all FCs. Buffer alone was injected after every 2 or 3 RBD
340 injections. The length of all injections was 30 s, and dissociation was monitored from 180-
341 670 s. The background response measured in FC1 was subtracted from the response in the
342 other three FCs. In addition, the responses during buffer injections closest in time were
343 subtracted from association and dissociation phases. Such double-referencing improves
344 data quality when binding responses are as low as needed to obtain accurate kinetic data
345 (Myszka, 1999). In each experiment, after all RBD injections, an ACE2-specific mouse
346 monoclonal antibody (NOVUS Biologicals, AC384) was injected at 5 µg/ml for 10 minutes to
347 confirm the presence and amount of immobilized ACE2.

348 **Data analysis**

349 Double referenced binding data was fitted using GraphPad Prism. The k_{off} was determined
350 by fitting a mono- exponential decay curve to data from the dissociation phase of each
351 injection. The k_{off} from four to six RBD injections was averaged to give a value for the k_{off}
352 (Fig. S3A). The k_{on} was determined by first fitting a mono-exponential association curve to

353 data from the association phase, yielding k_{obs}. The k_{on} was be determined by plotting the k_{obs}
354 vs the concentration of RBD and performing a linear fit of the equation $k_{obs} = k_{on}*[RBD] + k_{off}$
355 to this data (Fig. S3B), using the k_{off} determined as above.

356 The K_D was either calculated (calculated $K_D = k_{off}/k_{on}$) or measured directly (equilibrium K_D)
357 as follows. Equilibrium binding levels at a given [RBD]concentration were determined from
358 the fit above of the mono-exponential association phase model to the association phase
359 data. These equilibrium binding values were plotted against [RBD] and a fit of the simple
360 1:1 Langmuir binding model to this data was used to determine the equilibrium K_D (Fig. 2D).

361 ΔG for each affinity measurement was calculated the relationship $\Delta G = R*T*\ln K_D$, where R =
362 1.987 cal mol^{-1} K^{-1}, T = 310.18 K, and K_D is in units M. ΔΔG values (Table 2 and Fig. 3D) were
363 calculated for each mutant from the relationship $\Delta\Delta G = \Delta G_{WT} - \Delta G_M$. The predicted ΔΔG for
364 interactions with multiple mutants were calculated by adding the single mutant ΔΔG values
365 (Fig. 3D). The difference between the measured and predicted ΔΔG (ΔΔΔG) for interactions
366 between the ACE2 and RBD mutants was calculates as ΔΔΔG = measured ΔΔG – predicted
367 ΔΔG (Fig. 4B).

368 All errors represent standard deviations and errors for calculated values were determined
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376 Conflicts of interest

377 PAV owns BioNTech SE stock. The authors declare no other conflicts of interest.

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Table 1. Affinity and kinetic data for RBD variants and ACE2 variants

Mean and SD of the k_{off}, k_{on}, calculated and equilibrium K_D values for all RBD variants binding all ACE2 variants. For most measurements n = 3; the exceptions were WT RBD/ACE-2 equilibrium K_D measurements (n =24) and WT RBD measurements (n = 6). UK1, UK2, BR, SA refer to the B.1.1.7, VOC-202102-02, P2, and B.1.351 variants, respectively.

	k_{off} (s^{-1})	SD	k_{on} ($\mu M^{-1} s^{-1}$)	SD	K_D Calc. (nM)	SD	K_D Equi. (nM)	SD
RBD over WT ACE2								
WT	0.0668	0.00113	0.90	0.05	74.4	4.0	62.6	7.7
K417N	0.177	0.00416	0.49	0.05	364	29	349	10
K417T	0.126	0.00510	0.55	0.04	230	23	226	19
S477N	0.0348	0.00037	0.81	0.03	42.9	2.1	42.6	3.0
E484K	0.0818	0.00183	1.54	0.03	53.1	1.7	52.6	2.0
N501Y (UK1)	0.0111	0.00017	1.59	0.04	7.0	0.25	5.5	2.4
K417N/E484K	0.251	0.00799	1.02	0.07	247	23	251	23
K417T/E484K	0.168	0.00573	1.10	0.05	153	12	147	8.6
E484K/N501Y (UK2)	0.0118	0.00037	2.33	0.10	5.1	0.36	3.7	2.7
K417N/E484K/N501Y (SA)	0.0291	0.00076	1.46	0.06	20.0	0.70	17.4	3.1
K417T/E484K/N501Y (BR)	0.0211	0.00021	1.56	0.07	13.5	0.45	12.2	3.4
RBD over S19P ACE2								
WT	0.0298	0.00039	1.50	0.12	20.0	1.3	30.5	2.2
K417N	0.0782	0.00284	0.72	0.04	108	2.8	129	8.2
K417T	0.0521	0.00196	0.69	0.02	75.8	4.7	87.8	7.0
S477N	0.0257	0.00016	1.05	0.07	24.6	1.7	30.3	2.7
E484K	0.0325	0.00031	2.02	0.08	16.2	0.55	20.8	1.3
N501Y (UK1)	0.0051	0.00004	2.31	0.09	2.2	0.09	3.5	0.4
K417N/E484K	0.0961	0.00198	1.28	0.11	75.6	7.1	91.3	6.5
K417T/E484K	0.0660	0.00255	1.45	0.03	45.5	2.5	53.8	1.5
E484K/N501Y (UK2)	0.0051	0.00008	3.10	0.10	1.7	0.05	3.4	0.4
K417N/E484K/N501Y (SA)	0.0122	0.00009	2.16	0.03	5.7	0.07	10.4	1.2
K417T/E484K/N501Y (BR)	0.0085	0.00007	2.11	0.05	4.0	0.07	6.1	1.3
RBD over K26R ACE2								
S477N	0.0240	0.00009	1.07	0.05	22.6	1.1	33.4	1.3
WT	0.0500	0.00062	1.60	0.16	31.4	2.6	48.8	2.5
K417N	0.154	0.00789	0.88	0.07	175	8.1	237	15
K417T	0.101	0.00079	0.81	0.12	127	17.4	154	2.8
S477N	0.0240	0.00009	1.07	0.05	22.6	1.1	33.4	1.3
E484K	0.0587	0.00109	2.03	0.03	28.9	1.0	35.9	1.5
N501Y (UK1)	0.0081	0.00002	2.34	0.09	3.5	0.15	7.5	1.5
K417N/E484K	0.191	0.00481	1.48	0.15	130	9.4	166	11
K417T/E484K	0.135	0.00407	1.53	0.02	88.0	3.9	105	0.7
E484K/N501Y (UK2)	0.0085	0.00018	3.06	0.23	2.8	0.17	6.4	0.3
K417N/E484K/N501Y (SA)	0.0234	0.00040	2.13	0.05	11.0	0.28	18.7	2.0

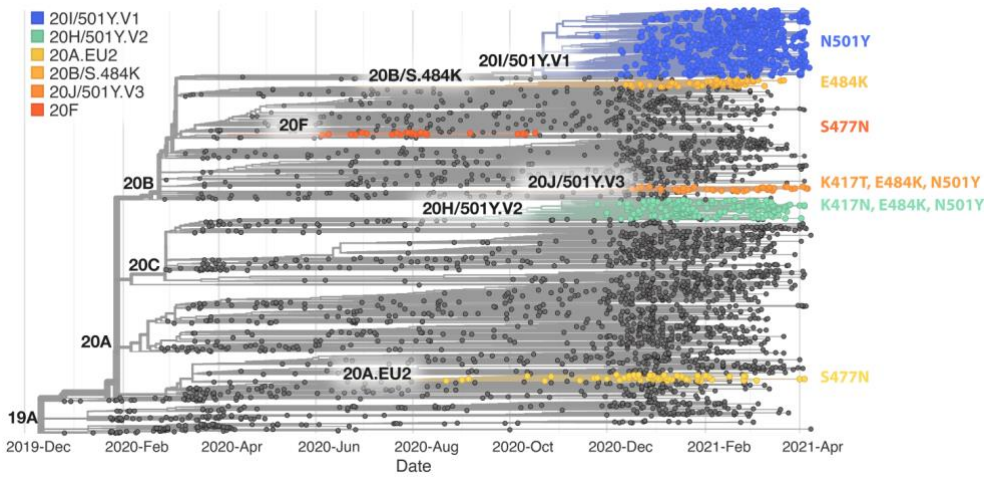
K417T/E484K/N501Y (BR)	0.0164	0.00028	2.21	0.06	7.4	0.33	15.3	0.8

Table 2. ΔΔG for RBD variants binding to ACE2 variants

Mean and SD of ΔΔG (n = 3, kcal/mol) were determined as described in the Materials and Methods using the calculated K_D values in Table 1. UK1, UK2, BR, and SA refer to the B.1.1.7, VOC-202102-02, P2, and B.1.351 variants, respectively.

	ACE2 WT		**ACE2 S19P**		**ACE2 K26R**	
RBD variant	**ΔΔG**	**SD**	**ΔΔG**	**SD**	**ΔΔG**	**SD**
WT	0.00	0.00	0.79	0.05	0.52	0.06
K417N	-0.96	0.06	-0.23	0.04	-0.52	0.04
K417T	-0.68	0.07	-0.01	0.05	-0.32	0.09
S477N	0.33	0.04	0.67	0.05	0.72	0.04
E484K	0.20	0.04	0.92	0.04	0.57	0.04
N501Y (UK1)	1.43	0.04	2.13	0.04	1.86	0.04
K417N/E484K	-0.72	0.07	-0.01	0.07	-0.34	0.06
K417T/E484K	-0.43	0.06	0.30	0.05	-0.10	0.04
E484K/N501Y (UK2)	1.62	0.05	2.30	0.04	1.98	0.05
K417N/E484K/N501Y (SA)	0.79	0.04	1.56	0.03	1.16	0.04
K417T/E484K/N501Y (BR)	1.03	0.04	1.76	0.03	1.39	0.04

A



B

	5	13	18	20	26	52	67	69	70	80	95	138	144	190	215	241	242	243	253	417	477	484	501	570	614	655	677	681	701	716	888	982	1118	1027	1176
																				RBD															
19A (Ref.)	L	S	L	T	P	Q	A	H	V	D	T	D	Y	R	D	L	L	A	D	K	S	E	N	A	D	H	Q	P	A	T	F	S	D	T	V
20I/501Y.V1 (B.1.1.7)	.	.	.	.	.	.	.	-	-	.	.	.	-	.	.	.	.	.	.	.	.	.	Y	D	G	.	.	H	.	I	.	A	H	.	.
20H/501Y.V2 (B.1.351)	.	.	F	.	.	.	.	.	.	A	.	.	.	.	G	-	-	-	.	N	.	K	Y	.	G	.	.	.	V	.	.	.	.	.	.
20J/501Y.V3 (P.1)	.	.	F	N	S	.	.	.	.	.	.	Y	.	S	.	.	.	.	.	T	.	K	Y	.	G	Y	.	.	.	.	.	.	.	I	F
20B/S.484K (P.2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	G	.	.	.	.	.	.	.	.	.	F
20A/S.484K (B.1.525)	.	.	.	.	.	R	V	-	-	.	.	.	-	.	.	.	.	.	.	.	.	K	.	.	G	.	H	.	.	.	L	.	.	.	.
20C/S.484K (B.1.526)	F	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	G	.	.	K	.	.	G	.	.	.	V.	.	.	.	.	.	.
20A.EU2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	G	.	.	.	.	.	.	.	.	.	.
20F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	G	.	.	.	.	.	.	.	.	.	.

C

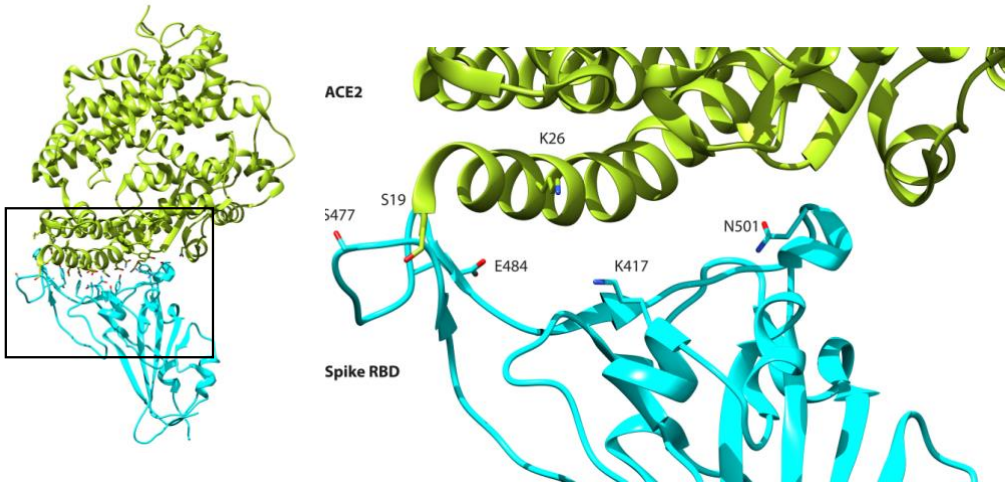


Figure 1. Spike RBD and ACE2 variants analysed in this study. (A) Phylogenetic tree illustrating the clades containing the RBD mutations investigated in this study. Constructed using TreeTime (Sagulenko et al., 2018) from the Nextstrain Global (Hadfield et al., 2018) sample of SARS-CoV-2 sequences from the GISAID database (Shu and McCauley, 2017) (Accessed 15th April 2021, N = 4,017). (B) Alignment illustrating the Spike residues that differ between SARS-CoV-2 variants, with the RBD mutants boxed. The variants are labelled with their clade designation from Nextstrain (Hadfield et al., 2018) and/or PANGO lineage (Rambaut et al., 2020)where relevant. The RBD mutations were collated from CoVariants

(Hodcroft, 2021) and Nextstrain. (C) The structure of SARS-CoV-2 Spike RBD (cyan) in complex with human ACE2 (green). The area enclosed by the box is shown enlarged in the right, with the residues mutated in this study labelled. Drawn using UCSF Chimera (Pettersen et al., 2004) using coordinates from PDB 6m0j (Lan et al., 2020).

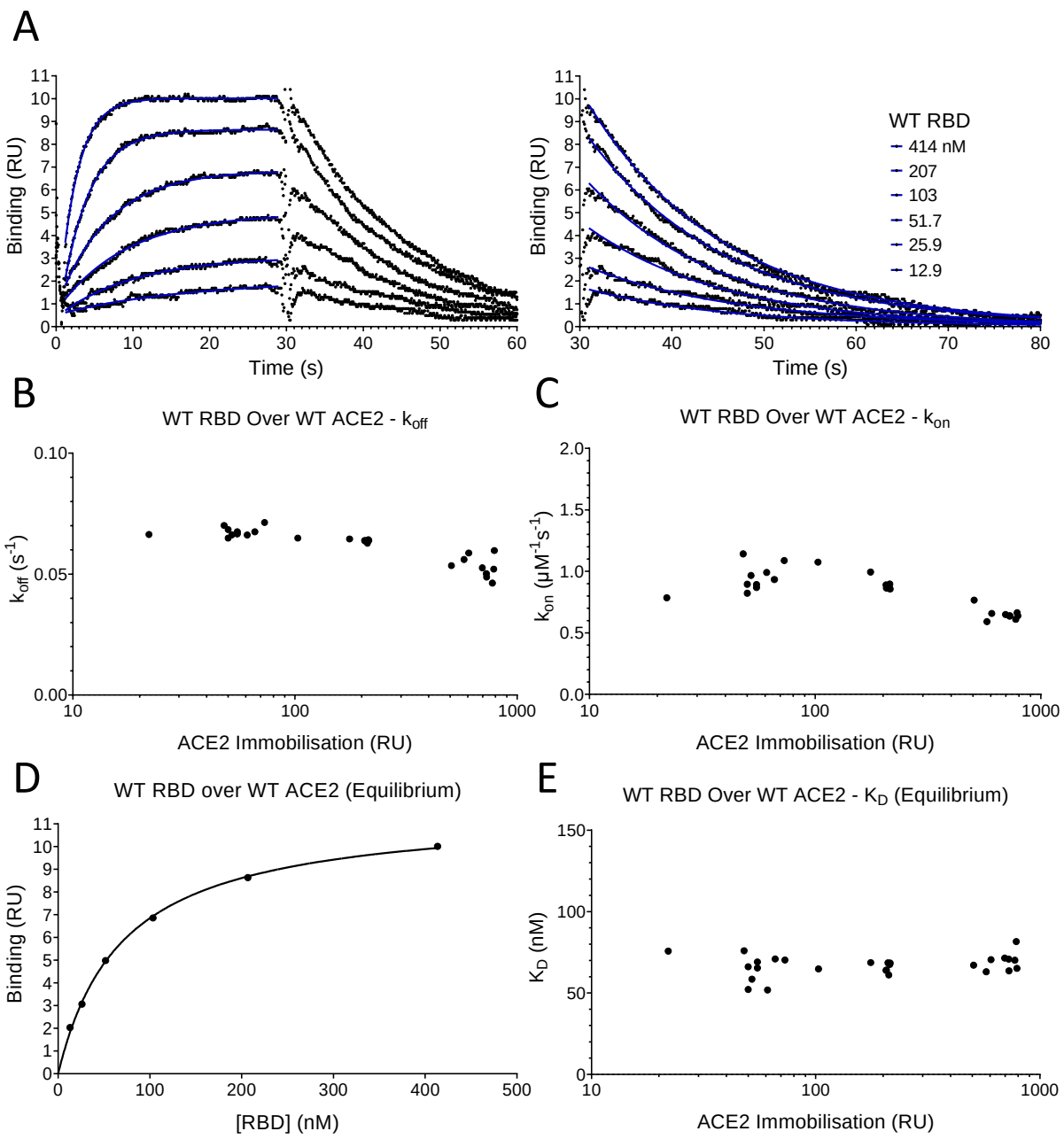


Figure 2. SPR analysis

(A) Overlay of binding traces showing association and dissociation when WT RBD is injected for 30 s at a range of concentration over immobilized WT ACE2. The right panel shows an expanded view of the dissociation phase. The blue lines show fits used for determining the k_{on} and k_{off}. The k_{on} was determined as described in Fig. S3. The k_{off} (B) and k_{on} (C) values measured at different levels of immobilized ACE2 are shown. (D) The equilibrium K_D was determined by plotting the binding at equilibrium against [RBD] injected. Data from experiment shown in A. (E) The equilibrium K_D measured at different levels of immobilized ACE2 are shown.

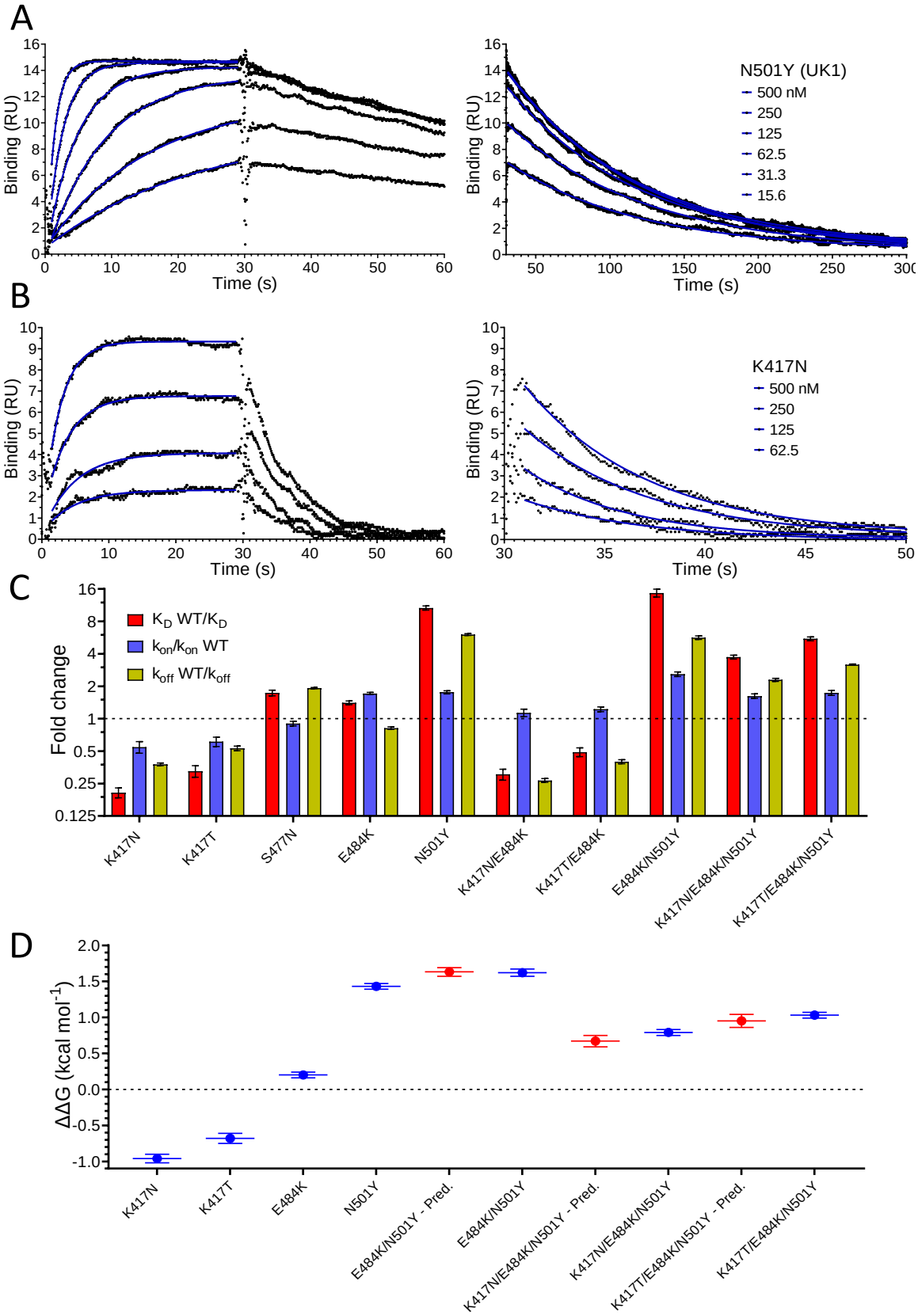


Figure 3. Effect of RBD variants binding WT ACE2

Overlay of binding traces showing association and dissociation of N501Y (A) and K417N (B) RBD variants when injected at a range of concentrations over immobilised WT ACE2. The

right panels show an expanded view of the dissociation phase. The blue lines show fits used for determining the k_{on} and k_{off}. (C) The fold change relative to WT RBD of the calculated $K_{D,}$ k_{on}, and k_{off} for the indicated RBD variants binding to immobilised WT ACE2 (Error bars show SD, n = 3). Representative sensorgrams from all mutants shown in Fig. S5, and the mean values from multiple repeats are in Table 1. (D) The blue lines show the measured ΔΔG for indicated RBD variants. The red lines show the predicted ΔΔG for the RBD variants with multiple mutations, which were calculated by adding ΔΔG values for single mutation variants (Error bars show SD, n = 3).

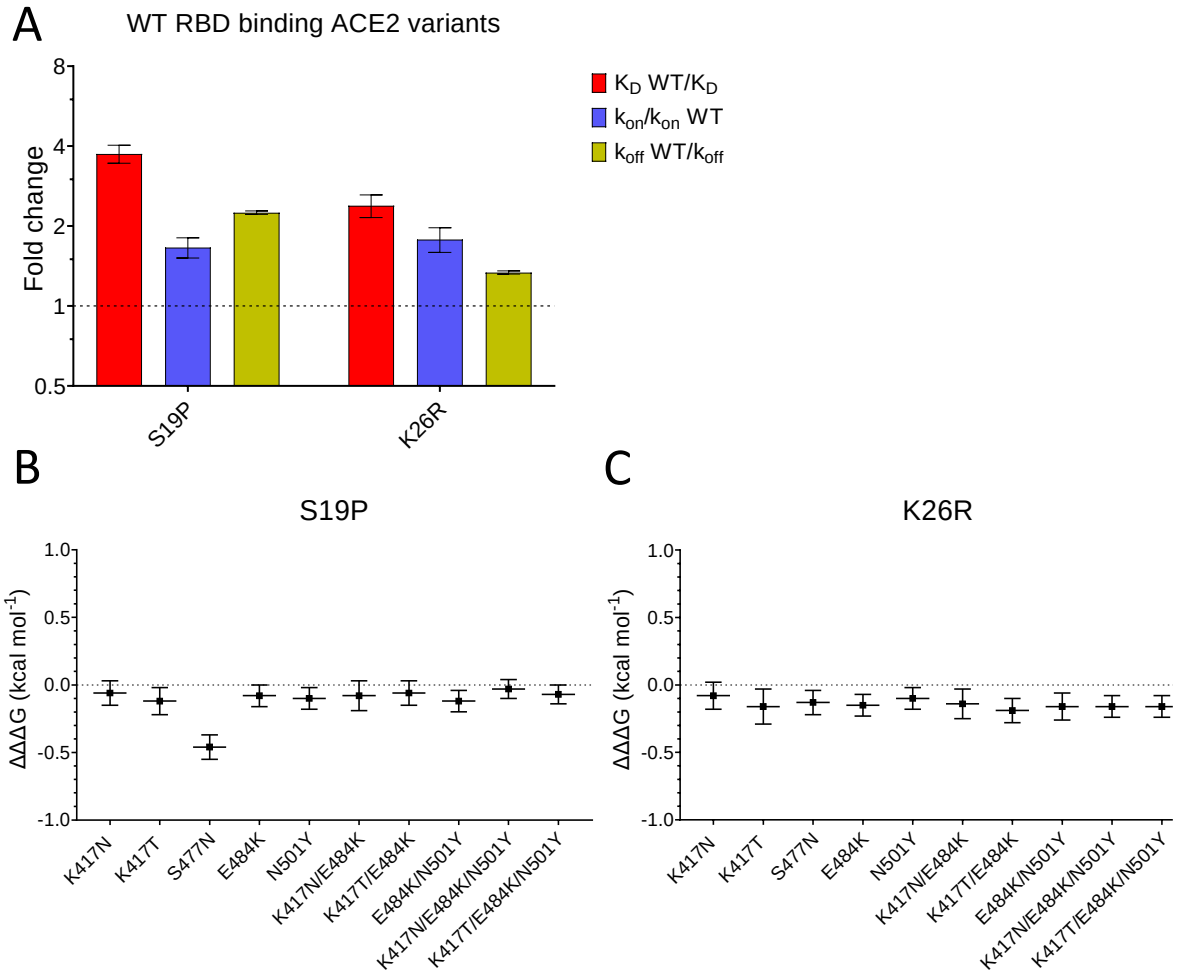


Figure 4. Effect of mutations in ACE2

(A) The fold change relative to WT ACE2 of the calculated K_D, k_{on}, and k_{off} for the interaction of WT RBD and the indicated ACE2 variants (Error bars show SD, n = 3). (B-C) Show the difference (ΔΔΔG) between the measured and predicted ΔΔG for S19P (B) and K26R (C) ACE2 variants binding to the indicated RBD variants, calculated from data in Table 2. The predicted ΔΔG values for each variant RBD/variant ACE2 interaction were calculated from the sum of the ΔΔG for the ACE2 variant binding WT RBD and the ΔΔG for the RBD variant binding WT ACE2 (Table 2).

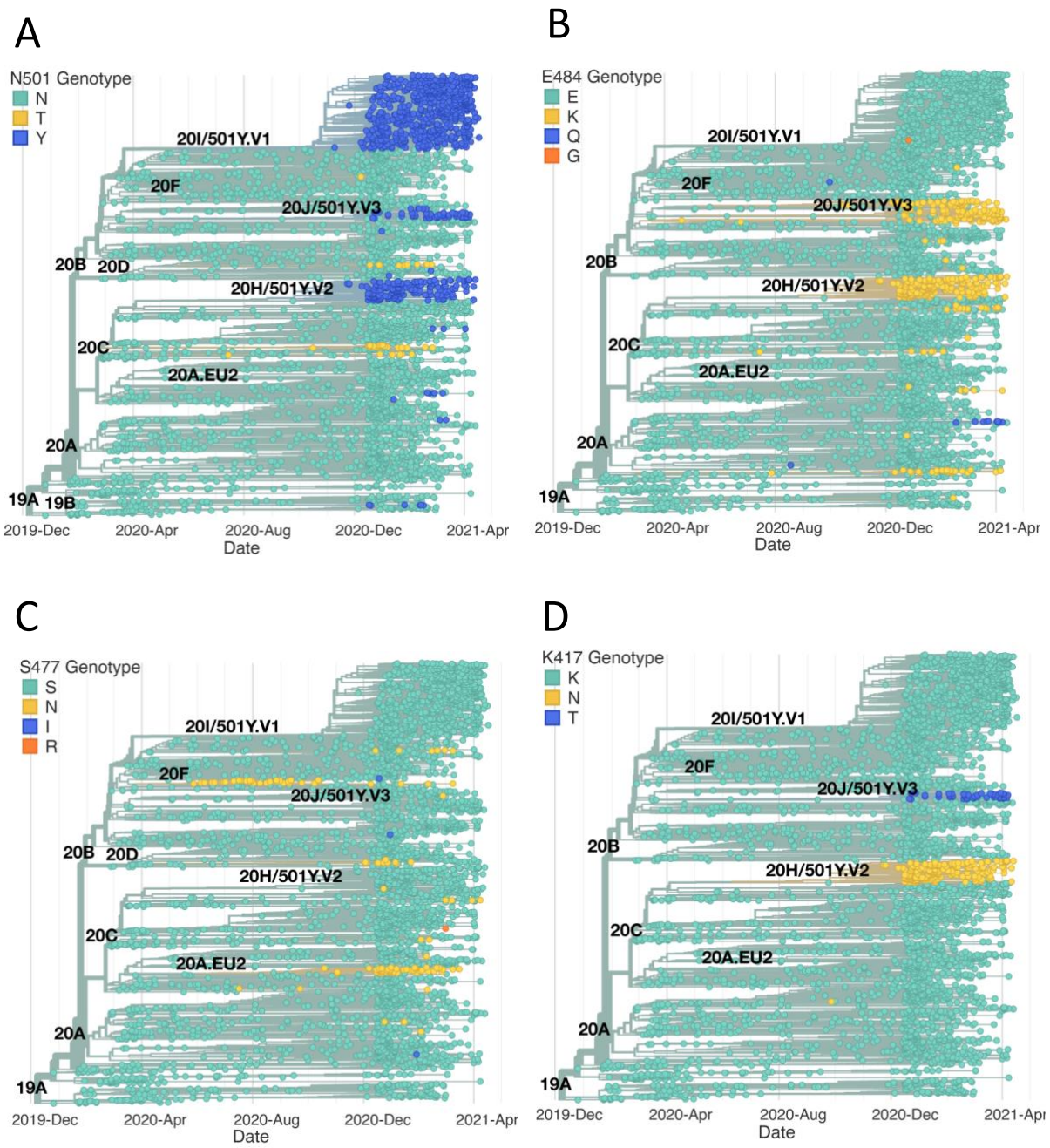


Figure S1. Emergence of the same RBD mutations in multiple SAR2-CoV-2 clades.
The figure highlights the SARS-CoV-2 clades containing RBD mutations investigated in this study. The phylogenetic trees were constructed as in Fig. 1A from SARS-CoV-2 sequences accessed on the 22nd April 2021 (N = 3,914). (A) N501Y has emerged independently of the three clades 501Y.V1, 501Y.V2, and 501Y.V3. Mutation to T at this position has also occurred frequently. (B) E484K has also been observed independently of its main progenitor clades 501Y.V2 and 501Y.V3. E484Q and E484G have also been observed. (C) S477N has been observed beyond clades 20F and 20A.EU2. Mutations to I and R have also been occasionally observed at this position. (D) Mutations of K417 to N and T have been observed almost exclusively in the 20H.501Y.V2 and 20J.501Y.V3 clades.

Figure S2

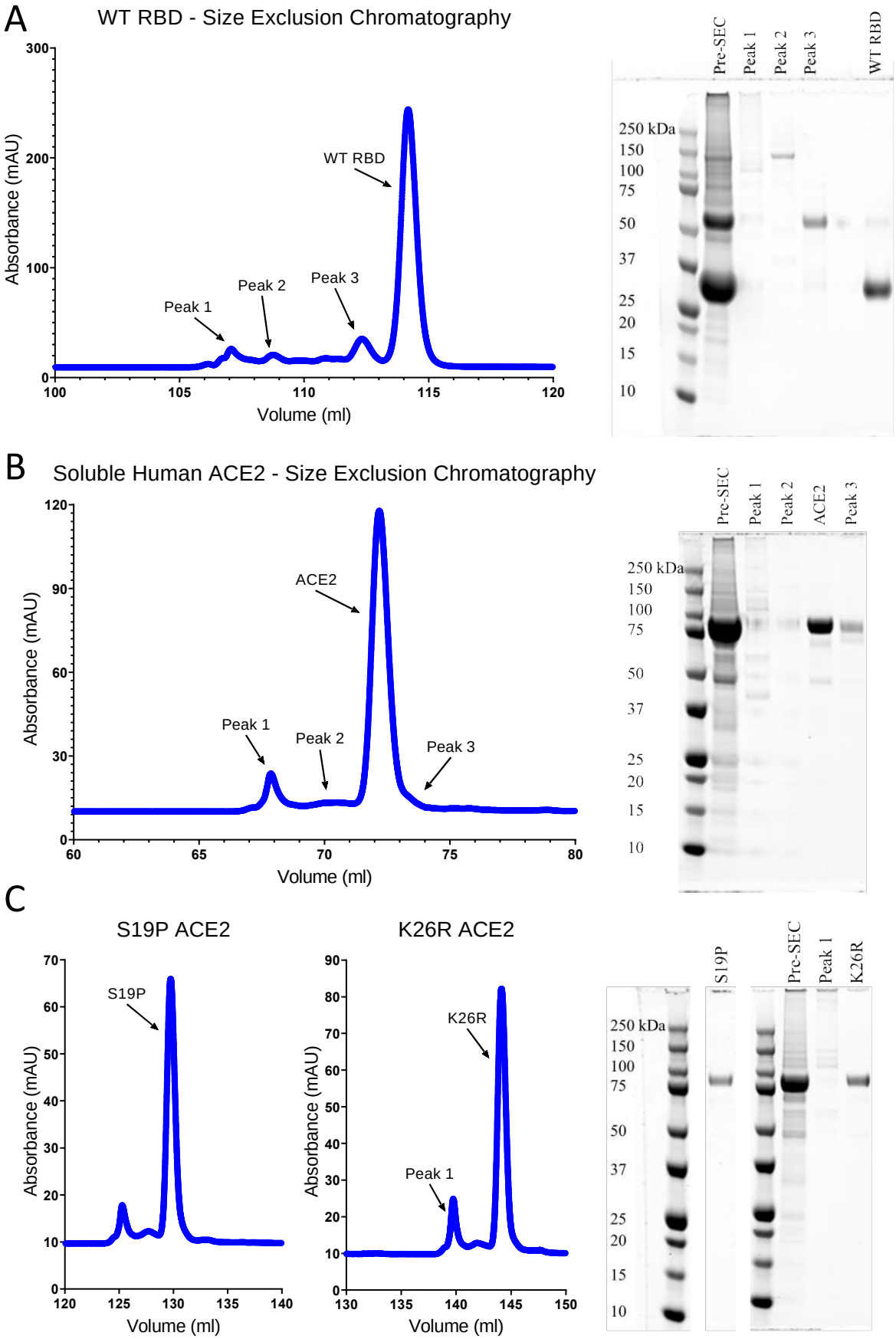
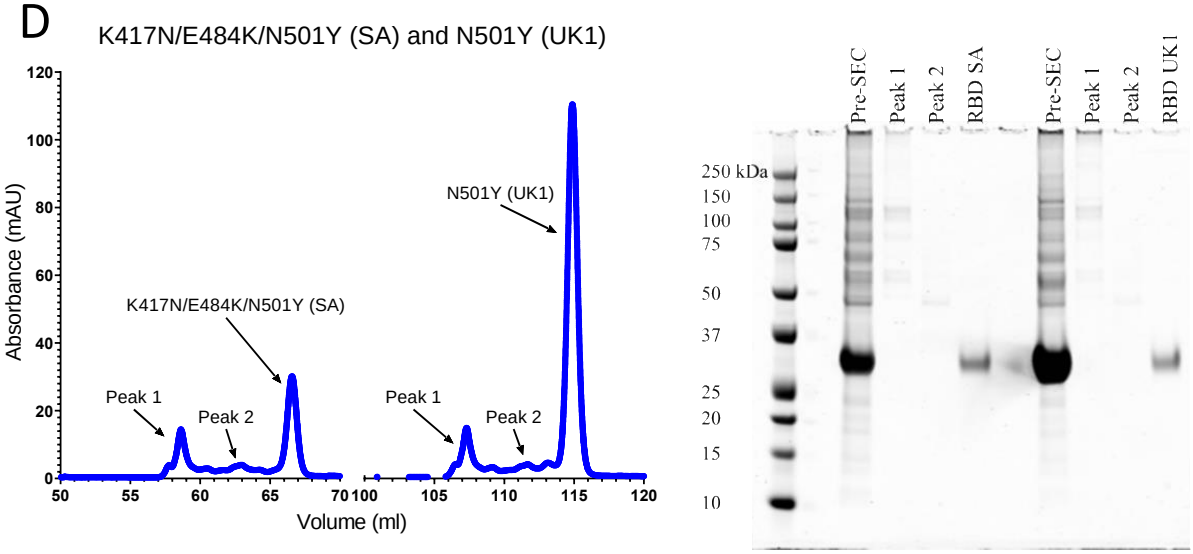

A
WT RBD - Size Exclusion Chromatography
Absorbance (mAU)
Volume (ml)
WT RBD
Peak 1
Peak 2
Peak 3
Pre-SEC
Peak 1
Peak 2
Peak 3
WT RBD
250 kDa
150
100
75
50
37
25
20
15
10
B
Soluble Human ACE2 - Size Exclusion Chromatography
Absorbance (mAU)
Volume (ml)
ACE2
Peak 1
Peak 2
Peak 3
Pre-SEC
Peak 1
Peak 2
ACE2
Peak 3
250 kDa
150
100
75
50
37
25
20
15
10
C
S19P ACE2
K26R ACE2
Absorbance (mAU)
Volume (ml)
S19P
K26R
Peak 1
S19P
Pre-SEC
Peak 1
K26R
250 kDa
150
100
75
50
37
25
20
15
10

Figure S2 cont

D



E

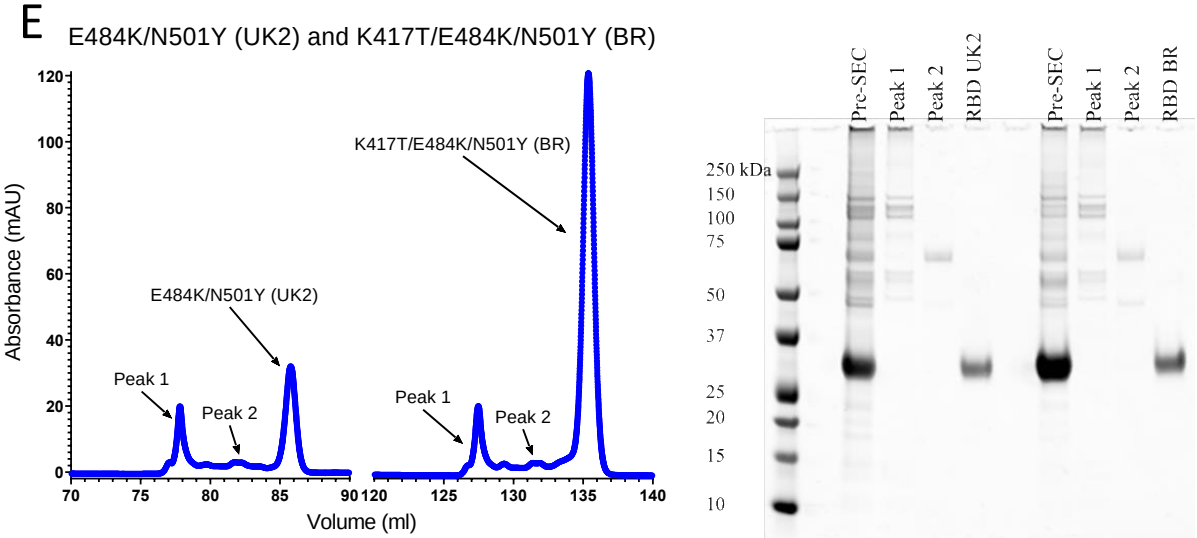


Figure S2 cont

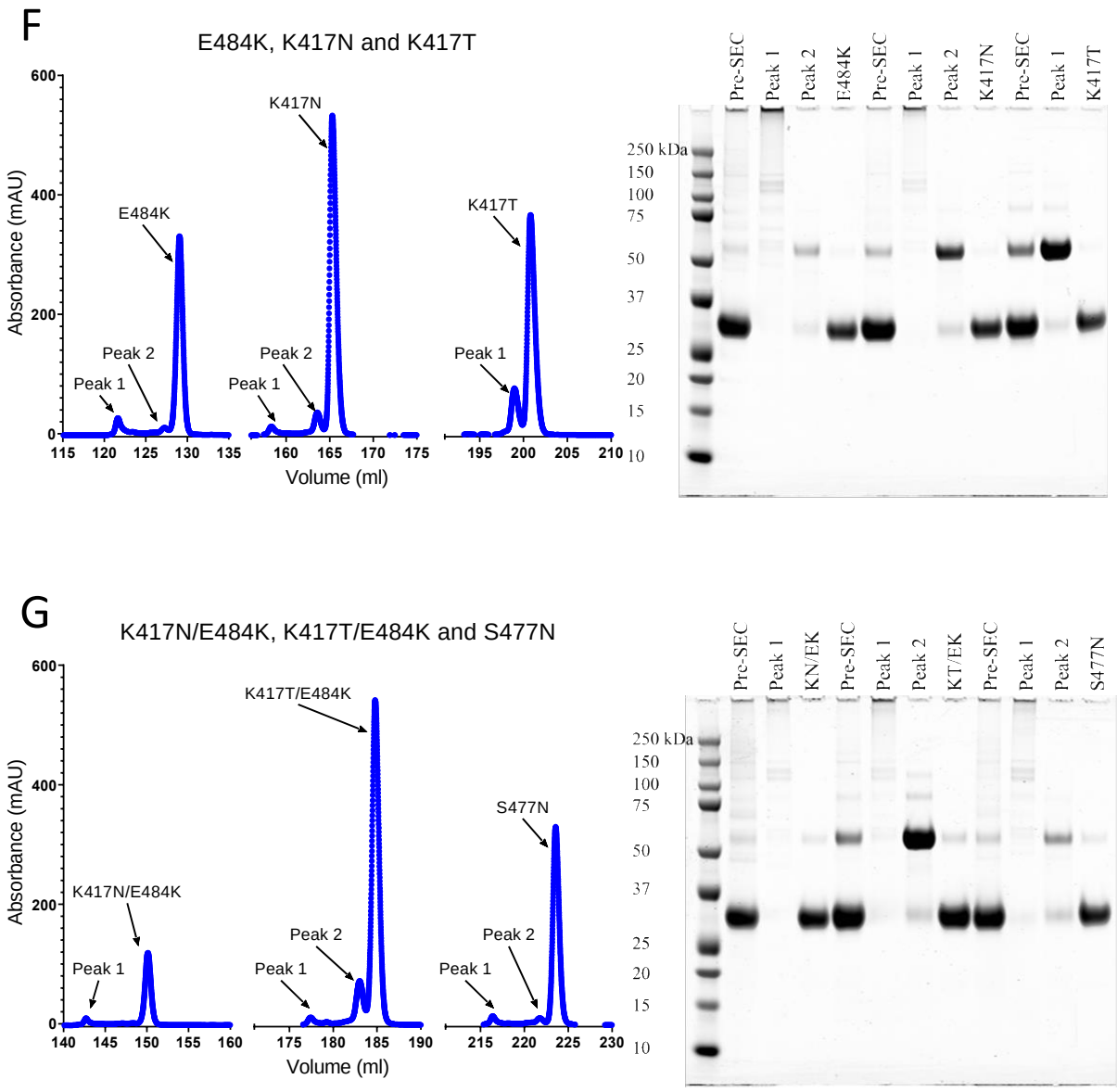


Figure S2. Protein purification

Size- exclusion chromatography traces of the indicated ACE2 and RBD proteins and SDS-PAGE of the indicated peak fractions. UK1, UK2, BR, SA refer to the B.1.1.7, VOC-202102-02, P2, and B.1.351 variants, respectively.

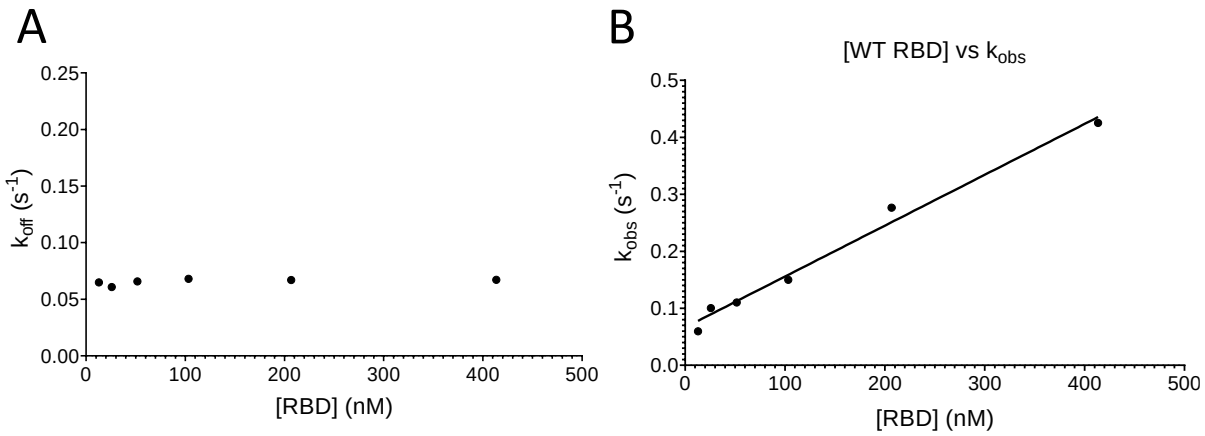


Figure S3. Determining the k_{on} and k_{off}.

Analysis of data from the fits in Fig. 2A. (A) A plot of k_{off} obtained for each injection versus [RBD]. (B) A plot of k_{obs} for each injection versus [RBD]. The constrained linear fit uses the average k_{off}, obtained in A, for Y-intercept. The k_{on} was obtained from the slope.

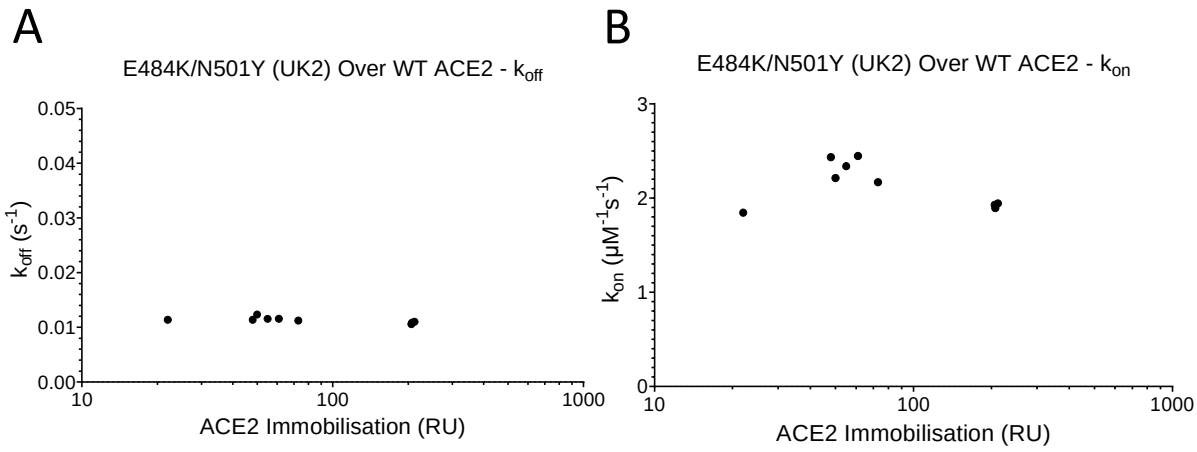
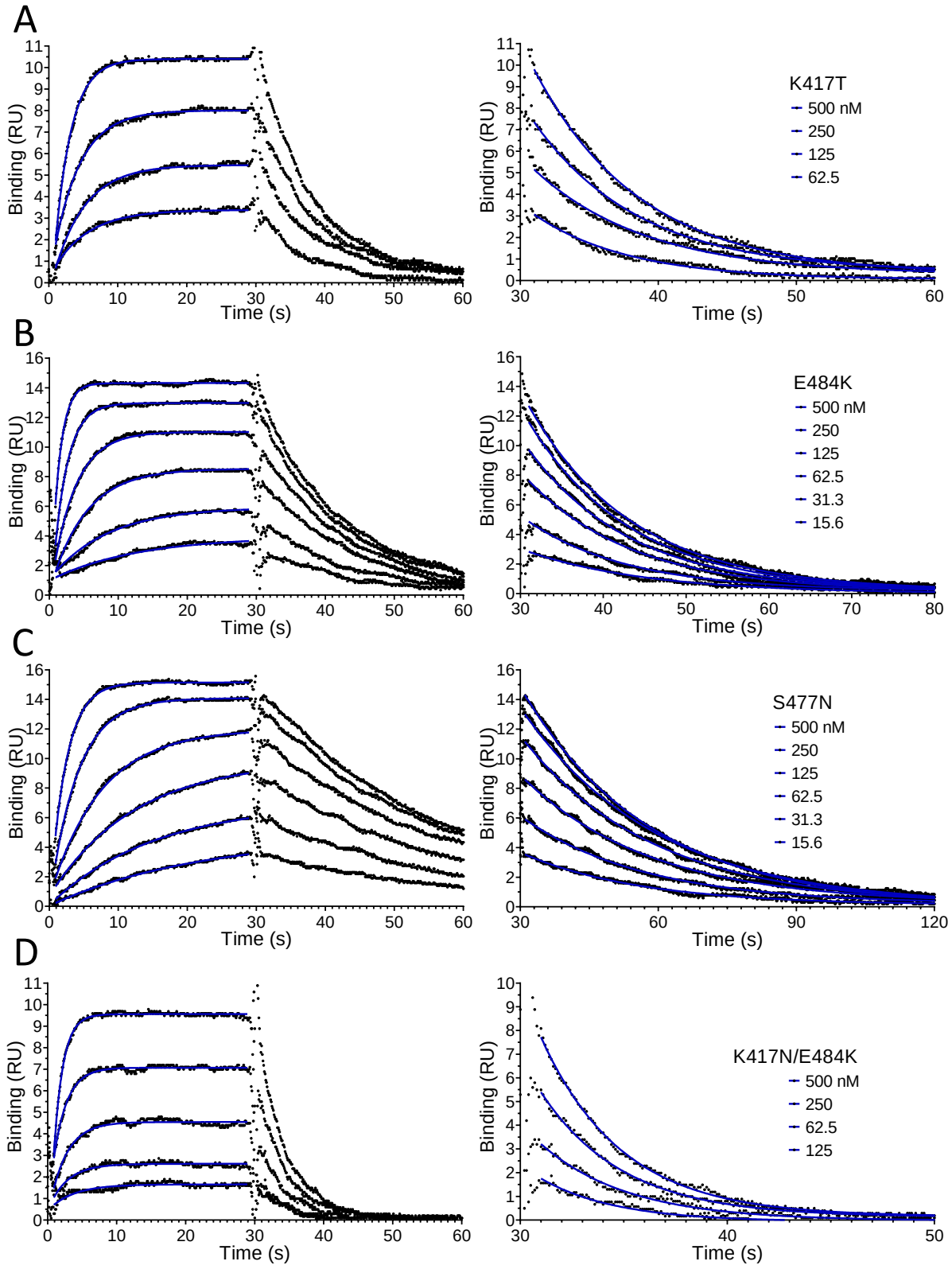


Figure S4. Mass transport controls from RBD

The k_{off} (A) and k_{on} (B), respectively, for E484K/N501Y (UK2) RBD binding WT ACE2 at a range of surface immobilisations (n = 12). UK2 refers to VOC-202102-02.

Figure S5


A
Binding (RU)
Time (s)
K417T
500 nM
250
125
62.5
B
E484K
500 nM
250
125
62.5
31.3
15.6
C
S477N
500 nM
250
125
62.5
31.3
15.6
D
K417N/E484K
500 nM
250
62.5
125

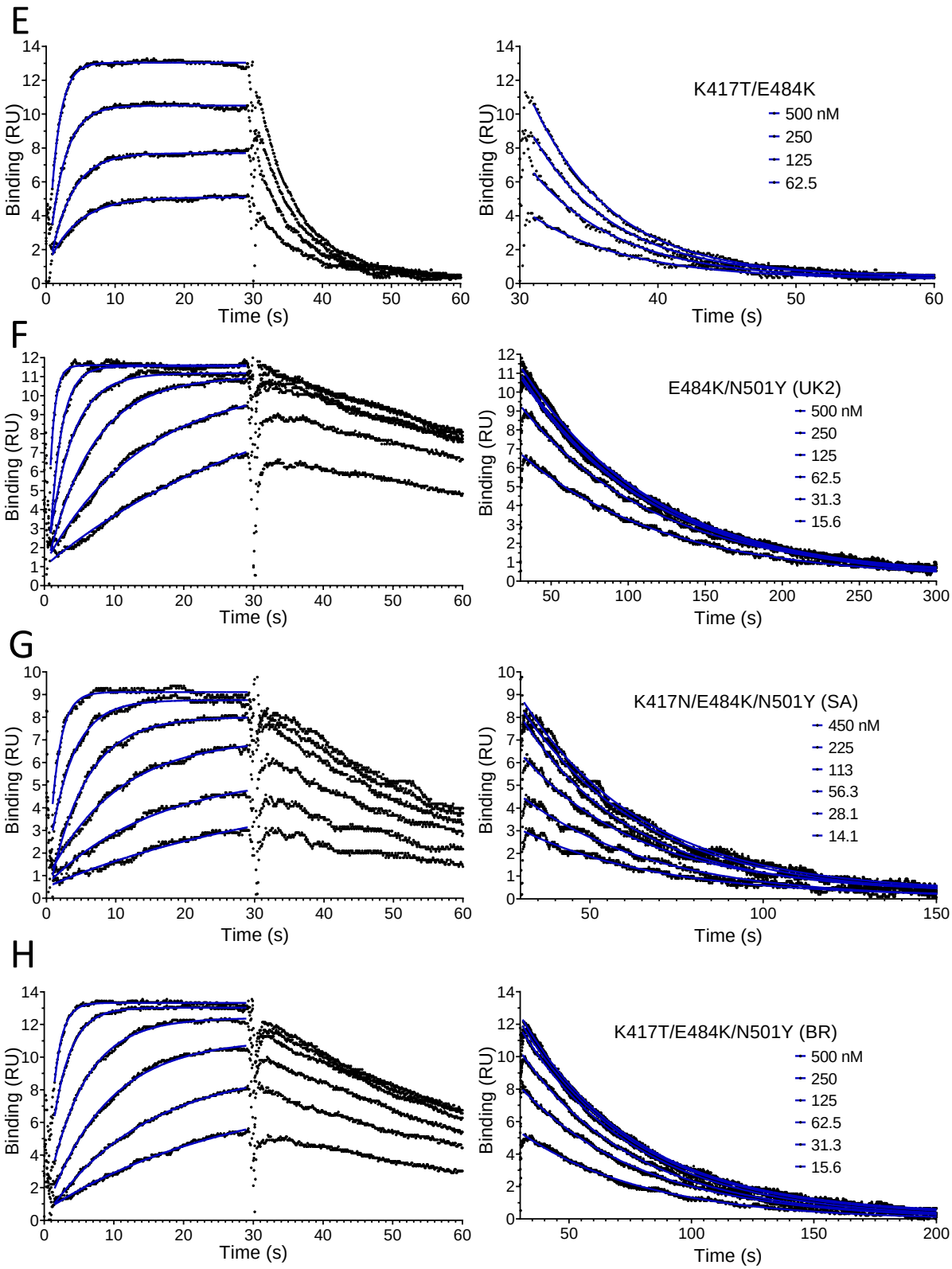


Figure S5. Representative SPR data for RBD variants binding to WT ACE2

Binding traces for the indicated RBD variants injected different concentrations over immobilised WT ACE2. The right panels show an expanded view of the dissociation phase. The blue lines show fits used for determining the k_{on} and k_{off}. UK1, UK2, BR, SA refer to the B.1.1.7, VOC-202102-02, P2, and B.1.351 variants, respectively.

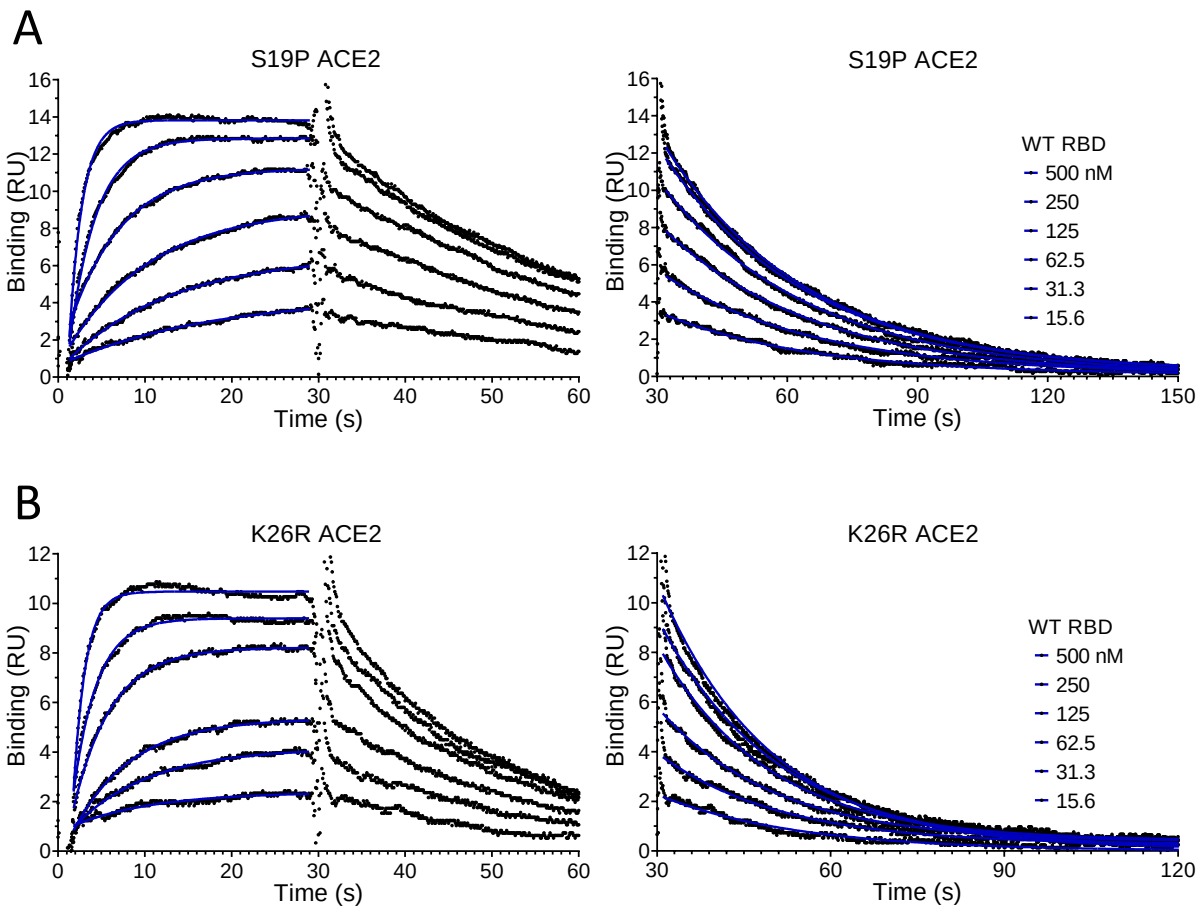


Figure S6. Representative SPR data for WT RBD binding ACE2 variants

Binding traces for the WT RBD injected at different concentrations over the indicated immobilized ACE2 variants. The right panels show an expanded view of the dissociation phase. The blue lines show fits used for determining the k_{on} and k_{off}.